Telomerase from *Saccharomyces cerevisiae* contains several protein subunits and may have different activities depending on the protein content

Andrey V. Petrov[a,b], Svetlana S. Dokudovskaya[a,c], Kirill A. Sokolov[a], Olga I. Lavrik[d], Alain Favre[c], Olga A. Dontsova[a,*], Alexey A. Bogdanov[a]

[a] *Department of Chemistry, Moscow State University, Moscow 119899, Russia*
[b] *Engelhardt Institute of Molecular Biology, Russian Academy of Science, Moscow, Russia*
[c] *Institut Jacques Monod, Paris, France*
[d] *Novosibirsk Institute of Bioorganic Chemistry RAS, Novosibirsk, Russia*

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Abstract Telomerase is a ribonucleoprotein responsible for maintaining telomeres during the cell cycle [1,2]. Here we describe a two-step purification procedure for the *Saccharomyces cerevisiae* telomerase complex. We have found that the properties (processivity, nuclease activity) of telomerase depend on the isolation procedure. Using a cross-linking approach, we have revealed several proteins that could be components of the telomerase complex. Furthermore, spectra of cross-linked proteins differ in processive and non-processive telomerase complexes.

Key words: Telomerase; Yeast; Processivity; Nuclease activity

1. Introduction

Telomerase is a multi-subunit enzyme which synthesizes GT-rich telomere repeats at the 3′-end of the DNA-chains in the vast majority of linear eucaryotic chromosomes by means of a reverse transcriptase reaction [1–4]. Telomerase differs from viral reverse transcriptase in at least two important features. RNA containing a template for synthesis of DNA-repeats is an integral part of telomerase [5]. After the completion of synthesis of one repeat, the catalytic subunit of telomerase has to start synthesis of the next repeat from the same template region of the telomeric RNA. Such a processivity has been shown for telomerases from different organisms [3,6,7]. Recently it was found [4,8,9], however, that a telomerase complex from *Saccharomyces cerevisiae* purified by ion-exchange chromatography can synthesize just a single repeat, and the DNA-primer remains tightly bound to the enzyme. On the other hand, it was shown [10] that a partially purified extract with telomerase activity from *S. cerevisiae* is able to synthesize telomeres consisting of several repeats, as efficiently as in the living cell. Thus, one may expect that this property of the telomerase complex depends on its protein content, which at the same time depends on the procedure used for telomerase purification.

In this paper we describe a two-step purification procedure for the telomerase complex from *S. cerevisiae*. We have found that this purification procedure yields a telomerase complex which (i) is processive (able to synthesize several telomeric repeats); (ii) has exonuclease activity; and (iii) can elongate non-telomeric primers. A cross-linking study has enabled us to demonstrate that the protein cross-linking patterns of the processive and non-processive enzyme differ significantly. Thus, we can suppose that the processivity of telomerase may be dependent on non-catalytic protein subunits of the telomerase complex.

2. Materials and methods

2.1. Crude extract preparation

Yeast extract was prepared as described [4,11]. Cells were harvested in early log phase (optical density at 600 nm was about 1) and the pellet was resuspended in buffer A (10 mM Tris-HCl, pH 7.8, 100 mM potassium glutamate, 1.2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM DTT, 5% (v/v) glycerol). After freezing the cells in liquid nitrogen they were subjected to mechanical disruption. After removing the cell debris by two centrifugations at $10\,000\times g$ (Beckman, J2-21) for 20 min at 4°C, the S-100 extract was prepared by ultracentrifugation at $100\,000\times g$ (Beckman L8-M) for 60 min at 4°C. The S-100 extract was concentrated about three-fold using Centricon columns 30 (Amicon).

2.2. Glycerol gradient purification

This step of yeast telomerase purification was performed as described [4] for the isolation of *Saccharomyces cerevisiae* telomerase complex. Ultracentrifugation was carried out in a 15–40% (v/v) glycerol gradient in buffer A at $200\,000\times g$ (Beckman L8-M) for 15 h at 4°C. The assay for telomerase in the gradient fractions was performed as follows: 10 mkl 5× concentrated extract was mixed with 10 mkl of 5× buffer TAB (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM DTT), 1 mkl of 10 mM dTTP, 1 mkl of [α-^{32}P]dGTP (3000 Ci/mmol), and 1 mkl of 50 mkM oligonucleotide 1 (5′-$(TG)_6$TGGG$(TG)_4$TGGG-3′), adjusted to 50 mkl with water. After incubation at 30°C for 1–1.5 h the reaction mixture was treated with RNase A (1 mkl of 10 mg/ml) for 30 min at 37°C. The DNA products were then extracted with phenol, ethanol precipitated, dissolved in the loading buffer (97.5% deionized formamide, 10 mM EDTA, together with 0.3% each of bromophenol blue (BPB) and xylene cyanol (XC)), and separated on a 10% denaturating acrylamide gel.

2.3. Ion-exchange chromatography

The extract partially purified by glycerol gradient centrifugation was fractionated on a DEAE-agarose column (Bio-Gel A Gel, Bio-Rad) [3]. After diluting the extract approximately 50–70 times with buffer B (10 mM Tris-HCl, pH 7.8, 1.2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM DTT, 10% (v/v) glycerol) it was loaded onto the column equilibrated with the same buffer. A linear gradient of NaOAc from 0 to 1000 mM was then applied. All fractions were collected, concentrated 8–10 times and desalted on Centricon columns 30 (Amicon) and used for telomerase assay.

*Corresponding author.

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2.4. Degradation of oligonucleotide

To examine the nuclease activity of the yeast telomerase complex oligonucleotides 1–9 were 5′-labeled with [α-^{32}P]ATP and T4 polynucleotide kinase and mixed with aliquots of the various column fractions. The reaction mixtures were incubated at 30°C for 30 min. The digestion products were resolved on 10% denaturing acrylamide gels.

2.5. Telomerase assay with non-telomeric primers

Different non-telomeric primers (2–9) were used to test the ability of the yeast telomerase to elongate these primers. The assay conditions were the same as those described above for telomeric primer 1.

2.6. Influence of the antisense oligonucleotide on the telomerase elongation reaction

Different amounts of 50 mkM solution of the antisense oligonucleotide (5′-GTGTGTGGGTGTGGTG-3′) which is completely complementary to the template region of telomerase RNA were incubated at 30°C for 15 min in 1× buffer TAB (10 mM Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 1 mM DTT) with 10 mkl of the extract partially purified by ultracentrifugation in a glycerol gradient. Then 1 mkl of 50 mkM stock solution of oligonucleotide 3 or 8 was added and the reaction mixture was incubated at 30°C for 1 h. The ratios of antisense oligonucleotide to tested oligonucleotide were 0, 0.5, 1, 2, 5, 10 and 50.

2.7. Cross-linking conditions

Ten mkl of column purified extract were mixed in 1× TAB buffer with 1 mkl of 50 mkM unlabeled primer 9, 1 mkl of 40 mM s^4dTTP, 2 mkl of 10 mM dATP, 2 mkl of [α-^{32}P]dGTP (3000 Ci/mmol). The reaction mixture was incubated for 30 min at 30°C. 50-mkl aliquots were spotted onto Parafilm stretched over an iced metal block and were irradiated for 10 min at 335 nm followed by 10 min at 365 nm. The reactions were then transfered to tubes containing 50 mkl of Laemmli gel loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.02% BPB) and incubated for 15 min at 56°C. The reaction products were resolved on 10% SDS-acrylamide gels for 17 h at 95 V.

3. Results and discussion

A cell-free extract prepared from *S. cerevisiae* strains 2085 and JD833, by the general procedure described for *E. aediculatus* and *S. cerevisiae* [4,11], was used for further purification of the yeast telomerase complex.

For preliminary purification of the enzyme complex we made use of ultracentrifugation in a 15–40% glycerol gradient. Telomerase activity was tested in each fraction, using the direct assay [3], based on the elongation of specific telomeric DNA-primer 1 by telomerase in the presence of [α-^{32}P]dGTP and dTTP. Fractions with telomerase activity were collected. Fig. 1A, lane 1, shows the activity corresponding to this sample. The position of the primer is marked by an arrow. Several rounds of elongation made by the gradient-purified telomerase can clearly be seen. No primer elongation was detected when the sample was treated with RNase A prior to the assay (lane 2), or in the absence of specific primer 1 (lane 3) or dTTP (lane 4).

For the second step of telomerase purification we used a DEAE-agarose column BioGel A Gel, which was eluted with 0–1000 mM linear gradient of NaOAc. The telomerase activity in the column fractions is shown in Fig. 1B and C. Two peaks of primer elongation activity corresponding to salt concentrations of 200–300 mM (Fig. 1B, lanes 4–8), and 700–900 mM (Fig. 1C, lanes 1–3) were detected.

In the first low salt peak of primer elongation, the elongation pattern changes with increasing salt concentration. The first fractions have a primer elongation pattern similar to that of the original glycerol gradient purified enzyme. In contrast, the telomerase complex in the following fractions loses the

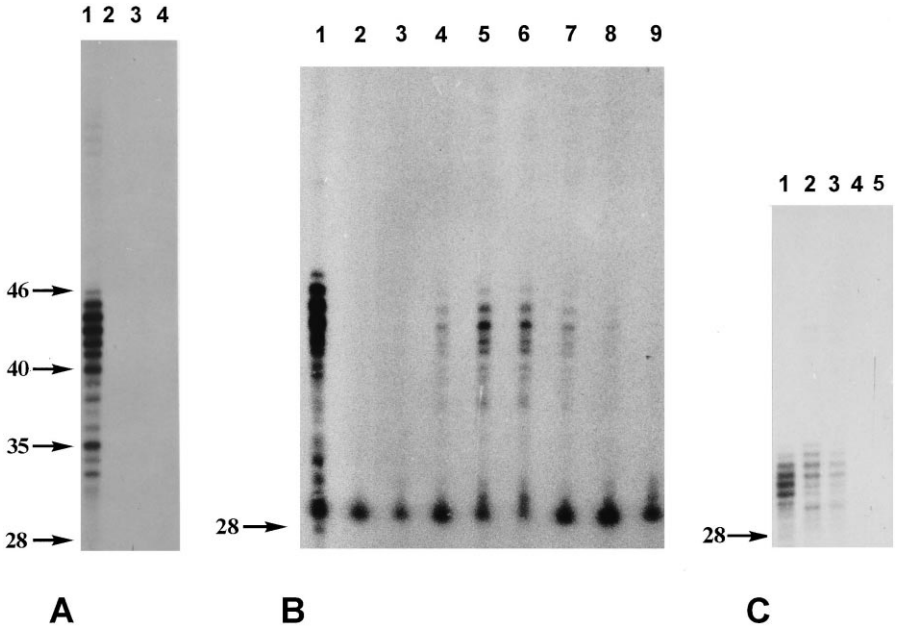

Fig. 1. Identification and characterization of *S. cerevisiae* telomerase purified by ultracentrifugation in a 15–40% glycerol gradient (A), followed by ion-exchange chromatography (B and C). Reactions were performed with primer 1, $(TG)_6TGGG(TG)_4TGGG$. A: Telomerase activity in glycerol gradient purified sample. Lane 1: Standard reaction (see Section 2); lane 2: pretreatment of extract with RNase A; lane 3: no input primer; lane 4: no input dTTP. B: Telomerase activity in the fractions corresponding to the first low-salt (200–300 mM) peak of primer elongation activity. Lane 1 (control lane): Assay with glycerol gradient purified telomerase; lanes 2–9: column fractions 4–11 accordingly. C: Telomerase activity in the fractions corresponding to the second high-salt (700–900 mM) peak of primer elongation activity. Lanes 1–5: Column fractions 15–19 accordingly.

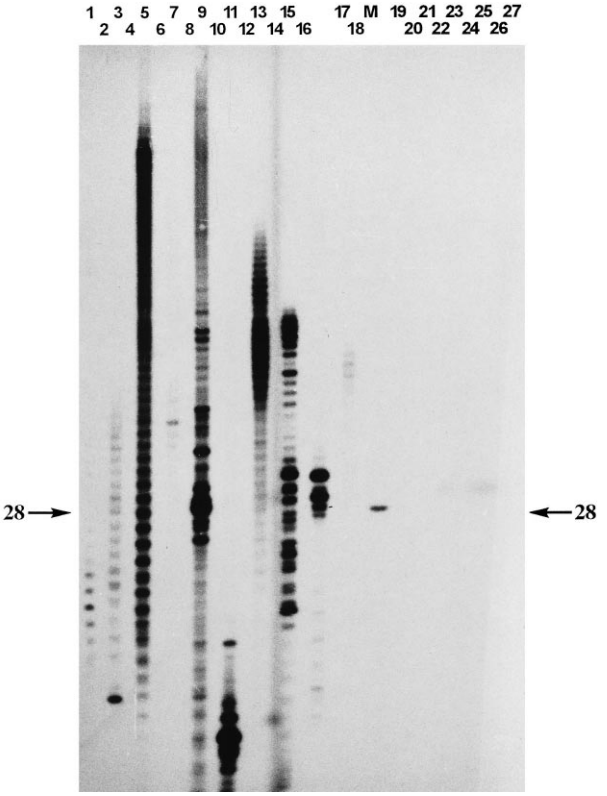

Fig. 2. Telomerase assay with different primers of the glycerol gradient purified enzyme. Odd lanes (1–17) correspond to the standard assay, even lanes (2–18) have no input dTTP. Lanes 1, 2: Oligonucleotide 2; lanes 3, 4: oligonucleotide 3; lanes 5, 6: oligonucleotide 4; lanes 7, 8: oligonucleotide 5; lanes 9, 10: oligonucleotide 6; lanes 11, 12: oligonucleotide 7; lanes 13, 14: oligonucleotide 8; lanes 15, 16: oligonucleotide 9; lanes 17, 18: oligonucleotide 1; lanes 19–27, RNase A pretreatment of extract for oligonucleotides 1–9 correspondingly.

processivity and can make only one round of elongation of the primer (Fig. 1B).

The second peak at higher salt concentration also shows telomerase activity. However, here the processivity is totally lost and the telomerase can elongate the primer only once (Fig. 1C). The latter observation is in good agreement with previous data [3,8,9,12,13] for a telomerase complex purified by ion-exchange column chromatography directly from a crude cell-free yeast extract.

Thus, we have found different telomerase complexes which possess different processivity. The properties of high-salt purified telomerase have been investigated in detail in the laboratory of E. Blackburn [3,8,9]. Our main goal was to study the properties and protein content of different fractions of the low salt telomerase peak, in which the enzyme is losing the processivity with the increasing salt concentration, as just noted.

It is well known that some telomerases [14–16] have exonuclease activity. We accordingly also tested the exonuclease activity of our telomerase complexes. For this purpose, oligodeoxyribonucleotides 1–9 were 5′-end-labelled with ^{32}P and added to the various column fractions. We found that only those fractions corresponding to the low salt telomerase complex have primer digestion activity. A similar picture was obtained for the glycerol gradient purified sample. We cannot be certain at the moment that our samples have 3′-exonuclease activity. However, according to data concerning other telomerases [15,16] it is unlikely that this primer digestion activity is due to an endonuclease or a 5′-exonuclease.

1. 5′-TGTGTGTGTGTGTGGGTGTGTGTGTGGG-3′;
2. 5′-CACCATCGAGTGCCTACC-3′;
3. 5′-GCCATCTATAAGTAGAAACAC-3′;
4. 5′-TTAGGGTTAGGGTTAGGG-3′;
5. 5′-CCGGAATTCCTCCCGATAGGTAGACC-3′;
6. 5′-GGGATCCTAATACGACTCACTATAGGAGCGGTCT-GAAAGTTA-3′;
7. 5′-CTCCTGCAGCCGGCCTGGCAGTTCCCTACTC-3′;
8. 5′-GGCAAGCTTAATACGACTCACTA-TAGGCCTGGCGGCCGTAGCGCGGTG-3′;
9. 5′-TTTTCACCCCATCTCCCTATAGTGAGTCGTATTA-3′.

We suggest that such exonuclease activity can be used by the telomerase for the elongation of non-telomeric primers. To test this suggestion several oligonucleotides were added to the telomerase complexes purified by glycerol gradient centrifugation and ion-exchange chromatography. We found that the oligonucleotides listed below can be processively elongated by telomerase.

The data for the glycerol gradient telomerase complex are shown in Fig. 2.

The most effective processive elongation was observed with oligonucleotide 9 (Fig. 2, lane 15). The yield of the reaction was much higher even than that seen with the 'true' telomeric oligonucleotide 1 (Fig. 2, lane 17). This oligonucleotide was chosen for further experiments.

It should be noted that all the primers listed above have

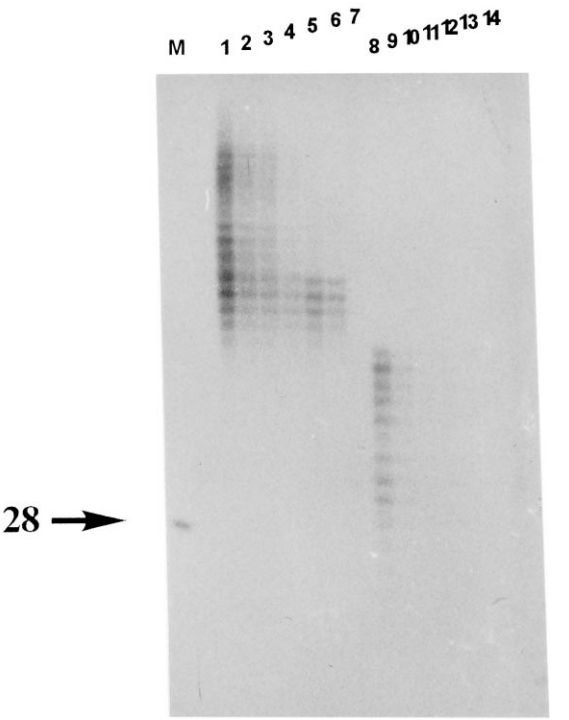

Fig. 3. Influence of antisense oligonucleotide on the telomerase elongation reaction. Lanes 1–7: Telomerase assay with primer 8; lanes 8–14: telomerase assay with oligonucleotide 3. Ratio antisense oligonucleotide/tested oligonucleotide was as follows: lanes 1, 8:0; lanes 2, 9:0.5; lanes 3, 10:1; lanes 4, 11:2; lanes 5, 12:5; lanes 6, 13:10; lanes 7, 14:50.

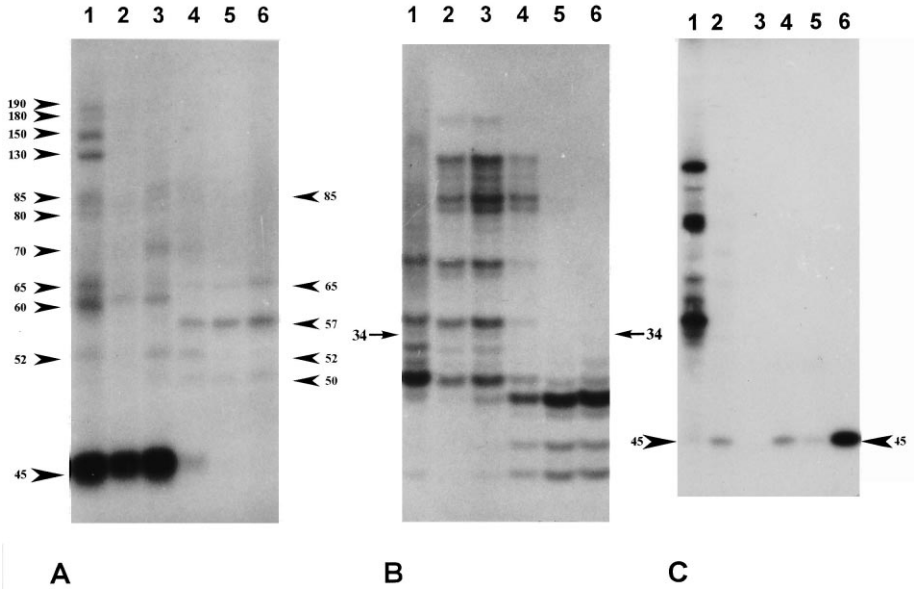

Fig. 4. Cross-linking experiment with different column fractions. A: Analysis of cross-linked proteins on a denaturing SDS-acrylamide gel. Lanes 1–6: Cross-links from the corresponding column fractions 6–11. B: Controls of primer elongation reactions for cross-linking. Lanes 1–6: Elongation patterns for the corresponding column fractions 6–11. C: Controls for specificity of the cross-linked proteins. Lane 1: Cross-linked proteins from combined fractions 6–11; lane 2: RNase A preatreatment; lane 3: dTTP instead of s^4dTTP; lane 4: no input s^4dTTP; lane 5: no input primer; lane 6: no UV irradiation.

GT, TG, or GTG motives in their sequences and, in accordance with the length of the shortest ^{32}P-labelled elongated oligonucleotide, the elongation reaction starts when these motives are at the very 3′-end of the oligonucleotides. Thus, we consider that the telomerase complex can digest the DNA-primer and select the proper sequence to start an elongation reaction. A similar effect was observed with the low salt telomerase fractions. The yield of the elongation reaction is increased in the presence of dATP and does not depend on rATP or rGTP. Again the processivity is lost with increasing salt concentration. No nuclease activity, and therefore no elongation of non-telomeric primer, was observed for a high salt telomerase complex (data not shown).

We found that the antisense oligonucleotide (5′-GTGTGGGTGTGGTG-3′), which is completely complementary to the template RNA region, cannot be elongated. We decided to test the influence of preincubation of the telomerase complex (glycerol gradient purified) with the antisense oligonucleotide on its ability to process non-telomeric primers. The data are presented in Fig. 3. Elongation of the relatively short primer 3 was found to be inhibited by preincubation with very low concentrations of the antisense oligonucleotide (Fig. 3, lanes 8–14). On the other hand, elongation of the relatively long primer 8 is affected in a different manner (Fig. 3, lanes 1–7). At low concentrations the antisense oligonucleotide specifically blocks the processivity and only a single round of elongation can be seen. Only high concentrations of the antisense oligonucleotide inhibit the reaction completely (Fig. 3). These data allow us to suggest that the telomerase anchor site for DNA binding [17] is essential for the elongation reaction and long oligonucleotides tightly bound to the telomerase can substitute for the shorter antisense oligonucleotide at the template region. At the same time the processivity is blocked. We can suppose that a protein subunit of telomerase specifically binds the GT-rich newly synthesized DNA-chain, thus preventing formation of a stable DNA-RNA duplex at the template region. This process could be essential for telomerase processivity, since it might be difficult for the enzyme to melt the duplex in order to move along the template region and start the synthesis of a new telomeric repeat. Thus, in the experiment with the antisense oligonucleotide this single-strand DNA-binding protein is blocked by the antisense oligonucleotide and only one elongation event takes place. We have observed that neither rATP nor rGTP affect the processivity. This observation supports our suggestion that, in order to start synthesis of a new DNA-repeat, the telomerase does not use an unwinding activity but rather binds the newly synthesized DNA chain, preventing duplex formation.

The data concerning processive and non-processive telomerase complexes obtained in this work allow us to suggest that these complexes could have different protein contents. To check this hypothesis we used a photoaffinity cross-linking approach.

This approach is based on the ability of the enzyme to incorporate a photo-analogue of an NTP into the growing nucleic acid chain. In our earlier studies [18] we have found that 4-thiouridine triphosphate is a very good substrate for RNA polymerase, and also is a very effective cross-linker. This residue can form cross-links both to proteins and RNA. It has also been shown [19] that 4-thio-thymidine triphosphate (s^4dTTP) is a good substrate for DNA polymerases. For these reasons we chose s^4dTTP as the photoreative moiety for our cross-linking study. We found that s^4dTTP in place of dTTP can be incorporated by telomerase into the growing DNA chain and that it produces a similar elongation pattern, although the level of incorporation is slightly decreased. Accordingly, s^4dTTP was added to the reaction mixture in place of dTTP, and the sample was subjected to mild (330–365 nm) UV irradiation. The cross-linked proteins were analyzed by gel electrophoresis. Oligodeoxyribonuclotide 9 was used as a primer because the elongation of this primer

is one of the most effective. In addition, this primer taking part in the cleavage-elongation reaction was used, to reveal as many telomerase protein subunits as possible that are responsible for the different telomerase activities. The cross-linking experiments were made with the low salt telomerase fractions, which differ in their processivity. The patterns of cross-linked proteins for the different fractions are shown in Fig. 4A, whereas the corresponding primer elongation spectrum is presented in Fig. 4B. The cross-linking pattern for the combined fractions 6–11 and different control lanes, which demonstrate the specificity of the cross-links, are shown in Fig. 4C. Comparison of the cross-linking patterns of processive and non-processive complexes shows that the spectrum of the cross-links differs significantly. In the processive fractions (Fig. 4A and 4B, lanes 1–4), cross-links to a protein of about 130 kDa (most probably the reverse transcriptase subunit) can clearly be seen. Efficient cross-links to other high molecular weight proteins are also observed. Cross-links to proteins of about 80 and 85 kDa are observed with lower efficiency, as well as to a protein of about 70 kDa. Other cross-links to proteins of about 52, 60 and 65 kDa occur with a high yield. In the non-processive telomerase complex (lanes 5–6), the yield of the cross-link to the high molecular weight proteins is significantly reduced, whereas the cross-links to the proteins of lower molecular weight become more efficient. The radioactive bands corresponding to other proteins with molecular weights lower than 50 kDa are present in the control lanes and most probably represent the tight binding of radioactive dGTP to some proteins. We cannot discriminate whether these low molecular weight proteins are telomerase specific or whether they are copurified with telomerase and other dGTP-binding proteins.

It should be noted that the cross-links were generated in the active telomerase. We can therefore not determine precisely how many proteins are cross-linked to the growing DNA chain, especially in the case of the processive complex, since DNA products of different length could participate in the cross-linking, thus giving cross-linked products of different molecular weights, but containing the same protein.

The most remarkable difference between the cross-linking patterns of the processive and non-processive complexes is the decrease in the cross-links to the higher molecular weight proteins in the latter case. It should be mentioned that the s^4dTTP is an effective cross-linker when it is incorporated into single-stranded DNA. In double-stranded DNA this reagent can only form cross-links with a protein that is located in the helix grove and in direct contact with the thio-group [20]. Thus, the reduction in cross-linking yield to the higher molecular weight proteins could be explained by the suggestion that in the non-processive telomerase complex an RNA-DNA duplex is formed, thus preventing a cross-link to the catalytic subunit and to the protein which can bind single-stranded DNA. On the other hand, the telomerase complex with arrested primer after one round of elongation could have a conformation which differs from that in the processive complex.

Our cross-linking data allow us to conclude that yeast telomerase has a very complicated structure and contains several protein subunits responsible for different activities. Rearrangement of the structure of this complex and changes in the subunit content presumably lead to the changes in the properties of the telomerase. The telomerase complex should have at least two enzymatic subunits: reverse transcriptase and exonuclease. Specific proteins may be responsible for the formation of an anchor site necessary for binding single-stranded DNA. Another protein might somehow bind the template region of telomerase RNA so as to keep the template region single-stranded and protect it from nucleases. Another protein could be responsible for binding the newly synthesized DNA chain during the elongation rounds, thus preventing a strong complementary interaction between DNA product and RNA template. This process could be particularly important for the enzyme processivity, since if such a duplex were to be formed it would be very difficult to destroy it and to translocate the

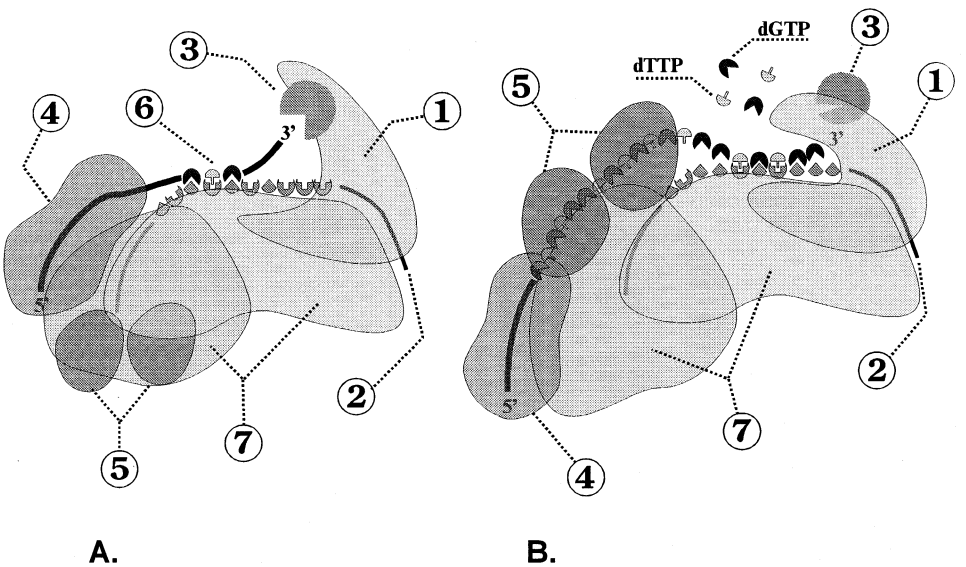

Fig. 5. Schematic presentation of the activities of the yeast telomerase complex. A: 3′-Degradation of primer to generate an optimal fit to the template region. B: Elongation reaction and binding of the DNA product by specific proteins. 1: Reverse transcriptase subunit (EST2); 2: telomerase RNA (TLC1); 3: nuclease subunit; 4: anchor site; 5: proteins specifically binding newly synthesized DNA chain; 6: template region; 7: other subunits.

RNA template along the DNA chain. A hypothetical scheme describing these interactions is shown in the cartoon (Fig. 5).

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